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Original Article

MiR-211 inhibits cell proliferation and invasion of gastric cancer by down-regulating SOX4

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Abstract: Introduction: Previous studies have shown that the dysregulation of miRNAs are frequently associated with cancer progression. Deregulation of miR-211 has been observed in various types of human cancers. However, its biological function in gastric cancer (GC) is still unknown. Methods: The expression of miR-211 in GC was detected by using quantitative real-time PCR (qRT-PCR). The miR-211 mimics and inhibitor were designed and transfected into BGC-823 cells. Then, we explore the probable biological function of miR-211 in gastric cancer cell proliferation and invasion in vitro. A luciferase reporter assay and western blot were performed to confirm the target gene of miR-211. Results: MiR-211 was significantly down-regulated in GC. Over-expression of miR-211 inhibited gastric cancer cell proliferation and invasion in vitro, conversely, down-regulated expression of miR-211 promoted gastric cancer cell proliferation and invasion. In addition, the sex-determining region Y-related high mobility group box 4 (SOX4) is identified as a target of miR-211 in GC cells, and SOX4 expression levels was inversely correlated with miR-211. Furthermore, knockdown of Sox4 inhibited the proliferation and invasion in GC cells. Conclusion: miR-211 could inhibit GC cell proliferation and invasion partially by down-regulating SOX4. MiR-211 might be a potential therapeutic target for GC treatment in the future.

Keywords: Gastric cancer, miR-211, SOX4, proliferation, invasion

Introduction

Gastric cancer (GC) is the most lethal type of gastrointestinal tract malignancy, and the third leading cause of cancer-related deaths in the world [1, 2]. It is estimated that approximately 1 million new cases of GC are diagnosed per year worldwide [3]. Although there have been recent advances in surgery and chemotherapy, the prognosis for GC remains poor [4, 5]. Therefore, a better understanding of the pathogenesis and molecular mechanisms involved in GC progression is essential to develop novel avenues for targeted therapy.

MicroRNAs (miRNAs) are small non-coding RNA molecules that bind to the 3'-untranslated region (UTR) of target mRNAs, resulting in translation repression or mRNA degradation, and play important roles in variety of biological events such as proliferation, development, differentiation, and apoptosis [6]. Previous studies have demonstrated that the dysregulation of these tiny and important molecules in different types of cancers including GC are frequently associated with cancer progression [7-9]. In recent years, many miRNA profiling studies have revealed that the expression level of microRNA-211 (miR-211) is abnormally expressed in various types of human cancers. MiR-211 expression was up-regulated in colon cancer, oral carcinoma, head and neck squamous cell carcinoma, the high expression of miRNA-211 was associated with poor prognosis of patients with these tumors [10-12]. On the other hand, the expression of miR-211 was down-regulated in epithelial ovarian cancer, hepatocellular carcinoma and breast cancer, playing a tumor suppressor role [13-15]. However, until now, the role and mechanism of miR-211 in GC have not been reported.

In this study, we aimed to determine the expression and function of miR-211 in GC. Furthermore, we explored the mechanism of miR-211 involvement in the development of GC by identifying

the possible target gene. Our study may provide a novel target for further studies of the therapy of Gastric cancer.

Materials and methods

Patients and samples

Human gastric cancer and their corresponding non-tumor tissues were collected at the time of surgical resection from 20 patients with gastric cancer from 2013 to 2014 at the Department of General Surgery, Huaihe Hospital of Henan University. Human tissues were immediately frozen in liquid nitrogen and stored at -80°C refrigerator. Signed informed consent was obtained from all patients and the study was approved by the Institute Research Ethics Committee of Huaihe Hospital of Henan University.

Cell lines and culture

Human Gastric cancer cell lines BGC-823, AGS, HGC-27, MKN-45 and the immortalized normal gastric epithelial cell line GES-1 were purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 U/mL penicillin (Invitrogen, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Cell transfections

The miR-211 mimics, inhibitor and non-specific miRNA negative control molecules were purchased from RiboBio Company (China). Small interfering RNA against SOX4 (si-SOX4) and negative control (si-NC) were designed by Genepharma Company (China). Transfection of cells was performed by using Lipofectamine™ 2000 (Invitrogen, USA) until a final concentration of 50 nM according to the manufacturer's instruction. After transfected and cultured for 48 h, cells were collected for Western blot and qRT-PCR analyses. Experiments were performed in triplicate.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay was used for cell proliferation analysis following the manufacturer's instruction. Cells were seeded in 96-well culture plates and incubated for 24, 48, 72 and 96 h at 37°C in a humidified atmosphere with 5% CO_2. The absorbance at 450 nm was then measured using a micro plate reader (BioTek, USA). All assays were independently performed in triplicate.

Cell invasion assay

The invasive potential of cells was measured by using Transwell invasion chambers coated with Matrigel (BD Biosciences, USA) according to the manufacturer's instruction. After being cultured for 48 h, cells were transferred into the upper chambers in serum-free RPMI-1640 medium. The lower chambers were added with RPMI-1640 containing 10% FBS. Cells were incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Then the invasive cells were on the lower surface were stained with crystal violet stain and counted by a light microscope. All experiments were repeated three times independently.

Quantitative real-time PCR

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen, USA) and then both miRNA and mRNA were reverse transcribed to cDNA. The reverse transcription was performed by using reverse transcription kit (Takara, Japan). Quantitative real-time PCR (qRT-PCR) were performed by using SYBR Green PCR Kit (Takara, Japan) on ABI 7500 Fast Real-Time PCR system (ABI, USA) according to the manufacturer's instructions. The expression of miR-211 was normalized to U6, SOX4 mRNA was normalized to GAPDH. All experiments were done in triplicate. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Total proteins were extracted and quantified using a protein assay (BCA method, USA). Proteins were fractionated by using 10% SDS-PAGE. The separated protein was transferred to PVDF membrane, blocked in 5% dry milk at room temperature for 1 hour and incubated with a primary antibody using anti-SOX4 and anti-GAPDH (Abcam, USA) followed by incubation with a secondary antibody. GAPDH was used as a control. Results were detected by the chemiluminescent detection system (Pierce ECL Substrate Western blot detection system,

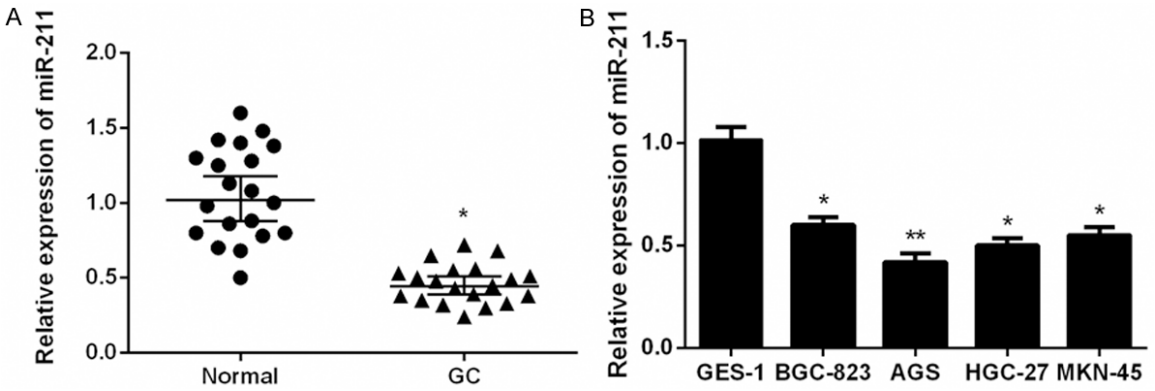

Figure 1. miR-211 is down-regulated in gastric cancer (GC) tissues and cell lines. A. The expression of miR-211 in 20 GC tissues and adjacent non-tumor tissues were examined by qRT-PCR. B. The expression of miR-211 in the immortalized normal gastric epithelial cell line GES-1 and GC cell lines (BGC-823, AGS, HGC-27 and MKN-45). *P < 0.05 and **P < 0.01.

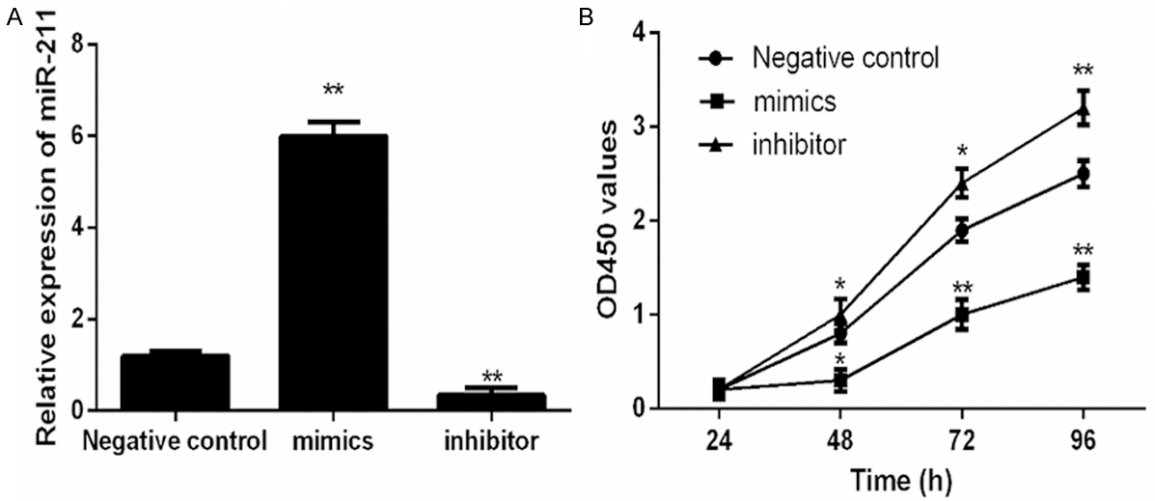

Figure 2. MiR-211 inhibits GC cell proliferation. A. MiR-211 mimics increase the expression of miR-211 and miR-211 inhibitor decrease the expression of miR-211 compared to miR-211 negative control in BGC-823 cells. B. CCK-8 proliferation assay showed that over-expression of miR-211 significantly inhibited the proliferation of the BGC-823 cells compared to the negative control. Conversely, miR-211 inhibitor promoted the proliferation of BGC-823 cells. *P < 0.05 and **P < 0.01.

USA). Each experiment was repeated independently three times.

Luciferase reporter assay

The 3'-UTR sequence of SOX4 which was predicted to interact with miR-211 or a mutant sequence with the predicted target sites were synthesized and inserted into the pGL3 promoter vector (Invitrogen, USA) named pGL3-SOX4-wt and pGL3-SOX4-mut. Cells were cultured in a 24-well plates and then co-transfected with miR-211 mimics or miRNA control, wide-type or mutant pGL3-SOX4 plasmid containing firefly luciferase, and the pGL3-SOX4 vector (Invitrogen, USA) containing renilla luciferase. Transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen, USA). Cells were collected 48 h after transfection and analyzed using the Dual Luciferase Reporter Assay (Promega, USA). All transfection experiments were repeated three times independently.

Statistical analysis

All statistical analyses were performed using SPSS 18.0. Data were presented as mean ± SD

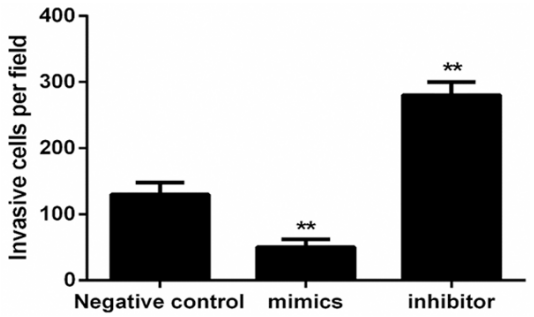

Figure 3. MiR-211 inhibits GC cell invasion. Transwell invasion assay demonstrated that over-expression of miR-211 markedly decreased the invasive capacity of BGC-823 cells compared to the negative control whereas miR-211 inhibitor promoted GC cell invasion. **P < 0.01.

from at least three independent experiments. Differences between groups were analyzed using student's t-test or one-way ANOVA analysis. A value of $P < 0.05$ was considered statistically significant.

Results

MiR-211 is down-regulated in GC tissues and cell lines

The expression of miR-211 in 20 paired GC tissues and non-tumor tissues were detected by qRT-PCR. We found that miR-211 was significantly down-regulated in GC tissues compared to non-tumor tissues (P < 0.05, **Figure 1A**). Moreover, the expression of miR-211 in GC cell lines (BGC-823, AGS, HGC-27 and MKN-45) was significantly decreased compared to the immortalized normal gastric epithelial cell line GES-1 (P < 0.05, **Figure 1B**).

MiR-211 inhibits cell proliferation

To study the role of miR-211 in the pathogenesis of GC, miR-211 mimics, inhibitor and the corresponding negative control were synthesized and transfected into the BGC-823 cells, respectively. The results demonstrated that the expression of miR-211 in BGC-823 cells transfected with miR-211 mimics was increased compared with cells transfected with the negative control, and the expression of miR-211 transfected with miR-211 inhibitor was decreased compared with cells transfected with the negative control (P < 0.05, **Figure 2A**). We then investigated the effect of miR-211 on cell proliferation of BGC-823 cells. As shown in **Figure 2B**, the proliferation rate of cells was markedly decreased by the transfection of miR-211 mimics compared to the negative control (P < 0.05). Conversely, miR-211 inhibitor significantly promoted the proliferation of the BGC-823 cells (P < 0.05).

MiR-211 inhibits cell invasion

To detect whether miR-211 affects the motility of GC cells, Transwell invasion assay was performed. Our results showed that miR-211 mimics significantly reduced the invasiveness of BGC-823 cells compared to the negative control whereas miR-211 inhibitor promoted the cell invasion ability (P < 0.05, **Figure 3**).

SOX4 is a direct target of miR-211

TargetScan was used to predict potential target genes of miR-211. We found that the 3'-UTR of SOX4 mRNA contained a target site for miR-211 (**Figure 4A**). To confirm SOX4 as a direct target of miR-211, luciferase reporter assay was performed. Our results showed that miR-211 significantly suppressed the luciferase activity of the wild type (wt) but not the mutant (mut) 3'-UTR of SOX4 (P < 0.05, **Figure 4B**). Moreover, western blot and qRT-PCR analyses showed that over-expression of miR-211 significantly decreased the expression of SOX4 in BGC-823 cells (P < 0.05, **Figure 4C**, **4D**). Taken together, these results indicated that SOX4 gene was one of the direct targets of miR-211.

SOX4 is inversely expressed with miR-211 in GC patients

The expression of SOX4 in GC tissues was significantly up-regulated compared to adjacent non-tumor tissues (P < 0.05, **Figure 5A**). Moreover, the expression of SOX4 in BGC-823, AGS, HGC-27 and MKN-45 GC cell lines was significantly higher than in the immortalized normal gastric epithelial cell line GES-1 (P < 0.05, **Figure 5B**). The expression levels of SOX4 was inversely correlated with miR-211 in GC patients (r=-4.124, P < 0.05, **Figure 5C**).

Effect of SOX4 on cell proliferation and invasion

To determine the role of SOX4 on GC cell proliferation and invasion, we performed knockdown of SOX4 expression by using small interfering RNA in BGC-823 cells. The expression of SOX4

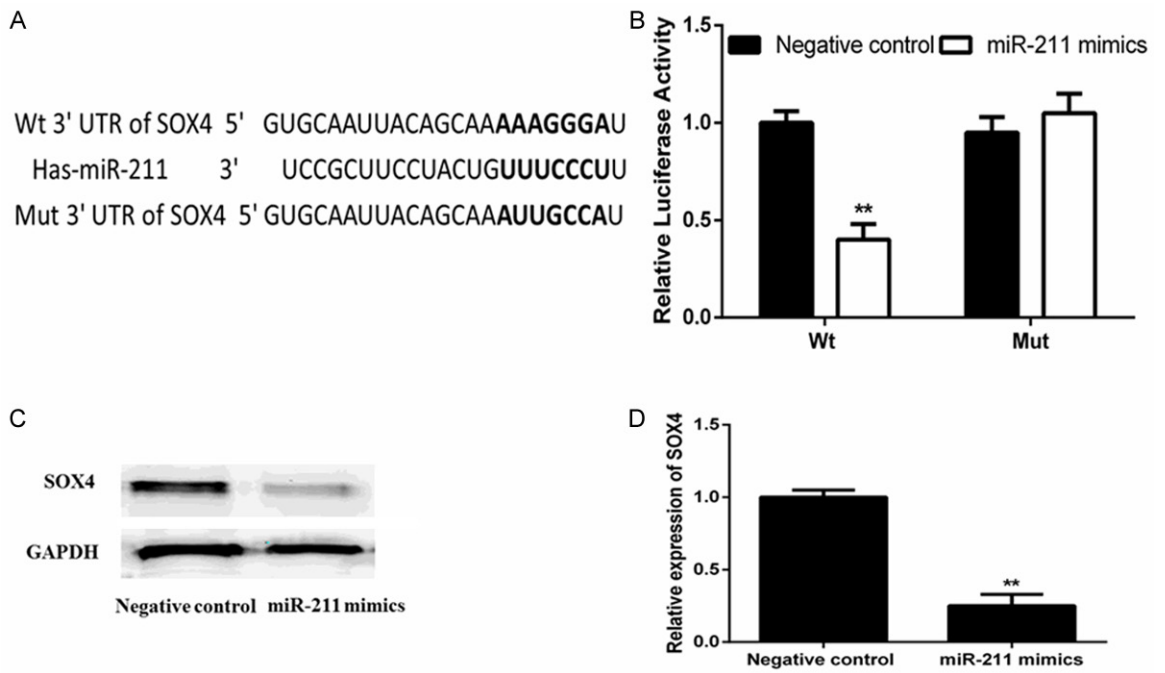

Figure 4. SOX4 is a direct target of miR-211. A. Computer prediction of the 3'-UTR of SOX4 mRNA contained a target site for miR-211. B. Luciferase activity assay revealed that miR-211 suppressed Wt SOX4 3'-UTR luciferase activity, while it had no effect on Mut SOX4 3'-UTR luciferase activity compared to control in BGC-823 cells. C. The protein level of SOX4 was detected by western blot after transfected with miR-211 mimics or control in BGC-823 cells. GAPDH was chosen as a loading control. D. The mRNA expression of SOX4 was examined by qRT-PCR analysis in BGC-823 cells. **P < 0.01.

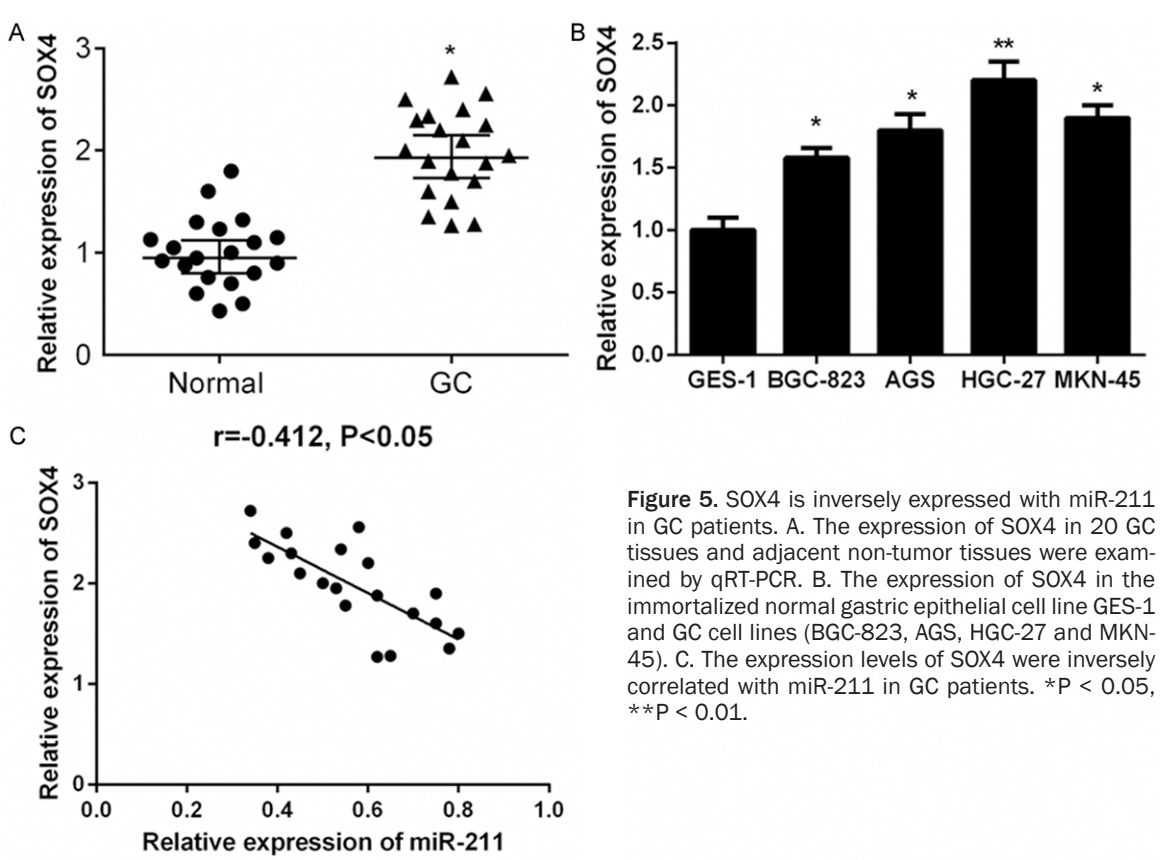

Figure 5. SOX4 is inversely expressed with miR-211 in GC patients. A. The expression of SOX4 in 20 GC tissues and adjacent non-tumor tissues were examined by qRT-PCR. B. The expression of SOX4 in the immortalized normal gastric epithelial cell line GES-1 and GC cell lines (BGC-823, AGS, HGC-27 and MKN-45). C. The expression levels of SOX4 were inversely correlated with miR-211 in GC patients. *P < 0.05, **P < 0.01.

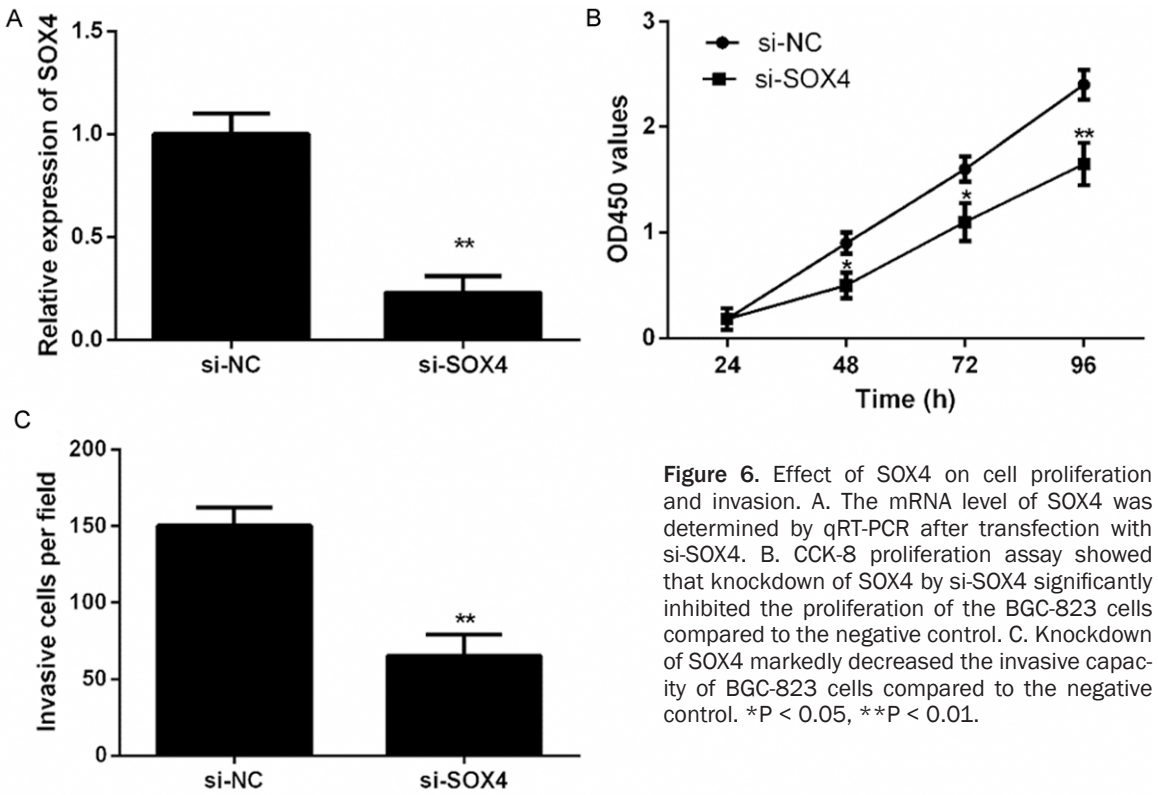

Figure 6. Effect of SOX4 on cell proliferation and invasion. A. The mRNA level of SOX4 was determined by qRT-PCR after transfection with si-SOX4. B. CCK-8 proliferation assay showed that knockdown of SOX4 by si-SOX4 significantly inhibited the proliferation of the BGC-823 cells compared to the negative control. C. Knockdown of SOX4 markedly decreased the invasive capacity of BGC-823 cells compared to the negative control. *P < 0.05, **P < 0.01.

transfected with si-SOX4 was significantly decreased compared with the negative control (P < 0.05, **Figure 6A**). As expected, knockdown of SOX4 inhibited the proliferation and invasion in BGC-823 cells compared to the cells transfected with si-NC (P < 0.05, **Figure 6B**, **6C**). These results suggested that knockdown of SOX4 play the similar effects as miR-211 overexpression, SOX4 is a functionally important target of miR-211.

Discussion

In this study, we investigated the roles of miR-211 in tumor proliferation and invasion of gastric cancer. We found that miR-211 expression was down-regulated in GC patients and cell lines compared to normal controls. Over-expression of miR-211 significantly inhibited the cell proliferation and invasion of GC cells, conversely, down-expression of miR-211 promoted the proliferation and invasion of GC cells. These results suggest that miR-211 may be a tumor suppressor in the development and progression of GC. We further investigated the molecular mechanisms whereby miR-211 inhibited the proliferation and invasion of GC cells. We identified that SOX4 is a direct target of miR-211 in GC cells by using bioinformatics and experimental assays. We found that the 3'-UTR of SOX4 mRNA contained a complementary sequence for miR-211. Over-expression of miR-211 significantly decreased the expression of SOX4 in GC cells.

The SOX4 (sex-determining region Y- related high mobility group box 4) gene is a member of SOX family of transcription factors, characterized by the important roles in embryonic development, cell fate determination, differentiation, and proliferation [16]. Recent studies demonstrated that SOX4 may contribute to the tumor progression. The expression of SOX4 was up-regulated in many types of human cancers, including non-small cell lung cancer, prostate cancer, colon cancer, and hepatocellular carcinoma [17-20]. Furthermore, over-expression of SOX4 enhanced proliferation of prostate cancer cells, and siRNA knockdown of SOX4 significantly decreased cell migration and invasion, suggesting an important role of SOX4 in cancer metastasis [21]. Song et al. found that SOX4 overexpression was an unfavorable prognostic factor in breast cancer patients [22]. Moreover, amount of studies showed miRNAs could play an important role in the regulation of SOX4. Liu

et al. demonstrated that miRNA-132 inhibited cell growth and metastasis in osteosarcoma cell lines by targeting SOX4 [23]. Yeh et al. also reported that miRNA-138 suppressed ovarian cancer cell invasion and metastasis by targeting SOX4 [24].

However, the expression and mechanism of SOX4 in GC are still unclear. In our study, SOX4 was upregulated in GC tissues; the expression of SOX4 in GC cell lines was also significantly higher than the immortalized normal gastric epithelial cell line GES-1. Moreover, knockdown of Sox4 inhibited the proliferation and invasion in GC cells compared with normal control. The expression of SOX4 was inversely correlated with miR-211 in GC patients. Our study demonstrated that SOX4 is a functional target gene of miR-211 in GC and over-expression of miR-211 is correlated with down-expression of SOX4 leading to the inhibition of GC cell proliferation and invasion. Taken together, miR-211 act as a tumor suppressor in gastric cancer by down-regulating SOX4, miR-211 might be a potential therapeutic choice for GC.

In conclusion, our study indicated that miR-211 was down-regulated, while SOX4 was up-regulated in gastric cancer. We demonstrated that SOX4 was a direct target of miR-211. Over-expression of miR-211 could inhibit GC cell proliferation and invasion by down-regulating SOX4. These results suggested that miR-211 might be a potential therapeutic target for GC.

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Disclosure of conflict of interest

None.

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